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DECLARATION UNDER 37 C.F.R 1.132

We, Radmila Mileusnic and Steven Peter Russell Rose, declare as follows:

1. We are members of the faculty of The Open University, Milton Keynes, MK7 6AA, United Kingdom, the assignee of the above-identified patent application. We are the inventors of the patent application.

2. We are also the co-authors, with Chris L. Lancashire and Amy N. Johnston, of the following publication, a copy of which is attached hereto: "APP is Required During an Early Phase of Memory Formation", *European Journal of Neuroscience*, 12(12):4487-4495 (December, 2000).

3. Chris L. Lancashire was employed by The Open University as a project officer and carried out routine assays under our supervision. Amy N. Johnston was employed by The Open University as a research fellow and carried out visualizations of the

CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231.

BY Dee Hatcher

DATE: July 12, 2002

antisense material to which reference is made in the specification. The work of Johnston was carried out at our direction.

4. Neither Lancashire nor Johnston contributed to the conception of the invention claimed in the patent application.

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further hat these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 25.06.02

RADMILA MILEUSNIC

Date: 25/06/02

STEVEN PETER RUSSEL ROSE

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APP is required during an early phase of memory formation

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Keywords: amnesia, APP antibody, APP antisense, APP328–332 peptides, β-amyloid precursor protein (βAPP), chick, memory

Abstract

The amyloid β/A4 protein precursor (APP) has been shown to be implicated in age-associated plastic changes at synapses that might contribute to memory loss in Alzheimer's disease. As APP has previously been reported to have multiple functions during normal development, we have employed a one-trial passive avoidance task in day-old chicks to study its role in the process of memory formation. Administration of anti-APP antibodies, injected 30 min pretraining, prevented memory for a one-trial passive avoidance task in day-old chicks without effects on general behaviour or initial acquisition. Amnesia was apparent by 30 min post-training and lasted for at least 24 h. The same result was obtained by down-regulation of APP expression by APP-antisense, injected 8–12 h pretraining. However, injections of anti-APP antibodies or APP antisense at later post-training time did not cause amnesia for the task. Unlike antibodies and antisense, injection of the APP328–332 pentapeptide, in either orientation, 30 min pretraining, rescued the memory and prevented antisense-induced amnesia. The post-training time within which the antibody- and antisense-induced amnesia, and within which the APP peptides prevent amnesia, correspond to that during which memory formation is vulnerable to disruption of the putative signal transduction functions of APP. These results suggest that: (i) APP is required during an early phase of memory formation, and (ii) the memory enhancing effect of secretory APP is localized within a 5-mer sequence of growth-promoting domain.

Introduction

The amyloid precursor protein APP, whose processing is disrupted in Alzheimer's disease, is a multifunctional transmembrane glycoprotein (Sandbrink *et al.*, 1994a,b; Selkoe, 1994). The high degree of evolutionary conservation of the extracellular and cytoplasmic domains of APP, its abundance in neurons and glia (Sandbrink, Masters & Beyreuther, 1994; Selkoe, 1994; Simons *et al.*, 1995; Storey *et al.*, 1996) and localization in postsynaptic densities (Shigematsu *et al.*, 1992) as well as in Notch-mediated signalling (Campos-Ortega, 1996) suggest important functions of APP in neuronal brain tissue.

APP may play a part in synapse formation and maintenance, but there is also evidence for a specific role in longer term memory formation (Doyle *et al.*, 1990; Flood *et al.*, 1991; Huber *et al.*, 1993; Müller *et al.*, 1994; Zheng *et al.*, 1995, 1996). APP knockout mice show impaired behavioural performance (Müller *et al.*, 1994; Zheng *et al.*, 1995, 1996). Blocking the extracellular domain of APP by intracerebral and intracerebroventricular administration of anti-APP antibodies targeted against various isoforms of APP differentially impair behaviour and memory in rats (Doyle *et al.*, 1990; Huber *et al.*, 1993). Smaller soluble fragments of β-amyloid (Aβ) and structurally mimetic nonpeptidic substances injected centrally antagonize the binding of Aβ protein and produce amnesia (Flood *et al.*, 1991) as well as a decrease of K^+-evoked acetylcholine release from hippocampus (Abe *et al.*, 1994; Maurice *et al.*, 1996). Centrally administered amyloid β peptides (Aβ) impair retention in the Y-maze, passive avoidance and place-learning in the water-maze (Maurice *et al.*, 1996), and cause amnesia for footshock active avoidance in mice (Flood *et al.*, 1991). Multiple bilateral injections of $A\beta_{1-40}$ into the dorsal hippocampus produce performance decrements in short-term working memory (Cleary *et al.*, 1995). In contrast to the effects of Aβ, the secreted form of APP (sAPP) when administered intracerebroventricularly shows potent memory-enhancing effects and blocked learning deficits induced by scopolamine (Meziane *et al.*, 1998).

Efforts to dissect the mechanism of physiological activity of APP resulted in the identification of the small stretch of amino acids containing the RERMS (NH_2-Arg-Glu-Arg-Met-Ser-COOH) sequence C-terminal to the KPI insertion site of sAPP-695 as the active domain responsible for growth promotion and neurite extension in neural cells (Ninomiya *et al.*, 1993; Jin *et al.*, 1994; Roch *et al.*, 1994), for neuronal survival (Yamamoto *et al.*, 1994) and for sAPP's ability to interfere with Aβ's deleterious effects on neurons (Li *et al.*, 1997). A synthetic peptide homologous to the RERMS sequence, APP328–332 peptide, is the shortest active peptide shown to exhibit trophic activity through cell-surface binding and induction of inositol polyphosphate accumulation (Jin *et al.*, 1994).

How APP is engaged in the molecular cascade underlying memory formation is, however, as yet unclear. Here we report, first, that anti-βAPP antibodies if injected intracerebrally around the time of training chicks on a one-trial passive avoidance task are indeed amnestic and, second, that antisense targeted against the transcription site −146 and the alternative initiation codon AUG_{1786} injected 6 or 12 h prior to training downregulates βAPP and causes amnesia 30 min after training. Finally, we demonstrate that the synthetic APP328–332 pentapeptide in both orientations (NH_2-RERMS and

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NH_2-SMRER), if injected 30 min before training, prevents the memory deficit induced by βAPP antisense administration. The results described above cast light on the role of two functional domains of APP–heparin-binding domain I and RERMS trophic domain and suggest that they might be mutually involved in memory loss in Alzheimer's disease. The use of biologically active peptides as a tool for the elucidation of APP's role in memory formation may point to a potential route for memory protection in neurodegenerative disorders.

Materials and methods

Animals and training

Commercially obtained Ross Chunky eggs were incubated and hatched in our own brooders and held until 16 ± 6-h-old. Chicks were then placed in pairs in small aluminium pens, pretrained and trained essentially as described by Lossner & Rose (1983). Following the last pretraining trial chicks were injected, where appropriate, as described below, before being trained by a 10-s. presentation of a 4-mm-diameter chrome bead which had been dipped in the bitter-tasting methylanthranilate (MeA). Chicks peck such a bead once, it elicits a disgust response and, if they remember the task, avoid similar but dry beads subsequently. At various times following training chicks were tested by offering them a dry 4-mm-diameter chrome bead followed 10 min later by a white bead, each for 10 s. Chicks were considered to have remembered the task if they avoided the chrome bead at test but pecked at the white bead (discrimination), and to have forgotten if they pecked at both beads. Only chicks that pecked at the bitter bead on training and the white bead at test were included in the final results. Amongst the chicks which met criterion (> 80%), recall was calculated as a per cent avoidance score (i.e. percentage of chicks in each group which avoided the chrome bead on test). Each chick was trained and tested only once and differences between groups were tested for statistical significance by the *g*-test (Sokal & Rohlf, 1981). The validity of this particular training task used to assess memory formation in this study is extensively discussed by Andrew (Andrew, 1991). All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Antibodies, oligodeoxynucleotides and peptide injections

Bilateral injections of anti-APP (1–5 μg in 2 μL; mAb22C11; Boehringer-Mannheim) antibody or saline controls were made using a 5-μL Hamilton syringe fitted with a plastic sleeve to allow a penetration of 3 mm. Chicks were injected into the left and right intermediate medial hyperstriatum ventrale (IMHV), a region known to be crucial in the early stages of memory formation for the task (for review see Rose, 2000). After completion of the injection, the needle was kept in place for 5 s. Correct placement was ensured by using a custom-built headholder (Davis *et al.*, 1979), and was routinely visually monitored postmortem. These injections are rapid (< 20 s per bird), do not require anaesthetic and cause no observable physiological or behavioural distress to the chicks. Chicks were tested at different time points post-training by an experimenter blind as to which treatment each chick had received.

Varying amounts of scrambled (SC) or antisense (AS) (0.6–1.0 μg) 16-mer end-protected phosphodiester oligodeoxynucleotide, 5′ CXC GAG GAC TGA XCC A 3′, designed to correspond to the transcription start sites –146 and AUG_{1786} of the βAPP mRNA, immediately upstream of a ribozyme binding site (King's College, Molecular Medicine Unit, London, UK), and of APP pentapeptides: RERMS (Bachem), SMRER and RSAER (MWG Ltd Biotech, Milton Keynes, UK) (0.5–5 μg) were injected intracerebrally at different time-points pre- or post-training.

In situ hybridization

The probes used for *in situ* hybridization were synthesized by King's College London (43–45 bases long, diluted to 5 ng/μL in DEPC-water). The APP sequence used was complementary to the positions 910–955 of the chick APP gene, described by Barnes *et al.* (1998). A control probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), corresponding to position 169– (45 mer; Domdey *et al.*, 1983) was used to control for nonspecific antisense effects. Method for *in situ* hybridization was as previously described (Morris, 1989). Probes were labelled with [^{35}S]dATP using terminal deoxyribonucleotidyl transferase (Promega) and purified using G50 microcolumns (Pharmacia). Brain sections were incubated with 200 μL of hybridization cocktail containing 1×10^3 cpm of the appropriate probe, covered and incubated in humidified Petri dishes with hybridization cocktail at 42 °C. Films were developed 5–10 days later in D-19 (Kodak) developer. Slides were routinely counterstained with cresyl violet.

Western blotting

Synaptic plasma membrane (SPM) proteins were prepared as described by Murakami *et al.* (1986), separated by SDS-PAGE under reducing conditions on a 10% polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose (Burnette, 1981). Blots were routinely checked by post-transfer staining with Ponceau S. The nitrocellulose was incubated in blocking buffer (Tris-buffered saline [TBS] pH 7.5 containing 5% defatted milk powder and 0.05% Tween 20). Antibodies were diluted in blocking buffer (5 μg/mL); the blot was incubated overnight at 4 °C and visualized with peroxidase-conjugated antimouse IgG.

Immunocytochemistry

Chicks were killed either 10 min, 60 min or 6 h after bilateral injection of mAb22C11 (by decapitation), and the forebrains removed, frozen slowly in precooled isopentane and stored at –40 °C. Frozen 15-μm cryostat sections were collected at –18 °C onto Super Frost Gold Plus slides (Merck), air dried for 30 min at room temperature and stored at –40 °C until processed. To locate the injected anti-APP, sections were probed with FITC-conjugated antimouse IgG (Vector Labs) diluted 1 : 50 at pH 7.5. To check that the injections were correctly placed, adjacent sections to the fluorescently probed ones were fixed and washed as above, then stained with 0.25% aqueous toluidine blue, dehydrated by passing through 80–100% ethanol and preserved using DPX mountant.

APP, GFAP and NeuN detection in ODNs- and APP328–332-treated animals

Chicks from the behavioural experiments were killed immediately following testing (by decapitation).

Forebrains were removed, frozen and cryostat sectioned, as described above. Sections were probed for APP, neuron-specific nuclear protein (NeuN) and glial fibrillary acidic protein (GFAP) using the Vector® MOM™ fluorescein immunodetection kit (Vector Labs). The anti-APP was identified by Texas Red-conjugated antimouse IgG, anti-GFAP was located by Cy3-conjugated antimouse IgG whilst the anti-NeuN was visualized with the Fluorescein Avidin DCS AMCA-conjugated antirabbit

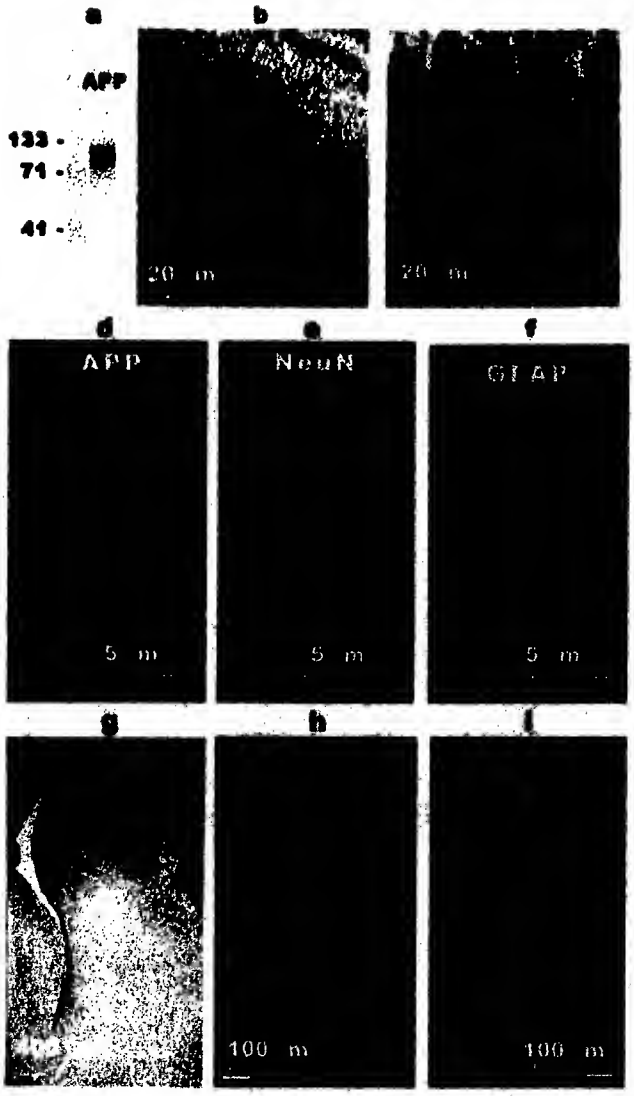

FIG. 1. βAPP in the chick brain. (a) Immunoreactivity of mAb22C11C with synaptic plasma membrane proteins isolated from 1-day-old chick brain. Lane one, Rainbow standards; Lane two, Western blot of 50 μg of SPM. (b and c) Cellular distribution of βAPP. (d–f) colocalization of (d) APP, (e) NeuN and (f) GFAP. (g) Toluidine blue counterstaining indicating (arrow) the injection site. (h) Ten minutes and (i) 60 min after injection, animals were decapitated, brain tissue was frozen and immunocytochemistry was carried out on 15-μm coronal sections as described in Materials and Methods. Sections were examined with a Leica DMR microscope.

IgG. Following the final wash, sections were preserved using a fluorescent antifade mountant.

Results

Detection and residence time of mAb22C11antibody in the chick forebrain

The Western blot in Fig. 1a shows that mAb211C recognizes a band in chick SPM running at 120 kDa. The cellular localization of the APP in the IMHV is shown in Fig. 1b and c. Cell phenotype was checked by NeuN and GFAP labelling (Fig. 1d–f). Anti-APP immunofluorescence was localized to axons and dendrites but there was no visible fluorescence of astrocytes as checked by colocalized GFAP.

To determine the pattern of binding of anti-APP antibodies in the chick IMHV, and their residence time therein, chicks were killed 10 or 60 min or 6 h following injection, brains frozen and prepared for immunocytochemistry. Figure 1g–i is a montage showing the injection site and the general appearance of the IMHV (Fig. 1g) and the distribution of immunofluorescence at 10 and 60 min after injection of the antibody (Fig. 1h–i). At the earliest time fluorescence appears around the injection site and trails down to the ventricles; by 60 min it is reduced, but still visible concentrated around the ventricles and by 6 h has vanished (not shown). The disappearance of fluorescence may be due to

inactivation, internalization or migration of the antibody; for whatever reason the biologically efficacious residence time of the antibody in the IMHV is relatively short.

Effects of mAb22C11 on learning and memory for the passive avoidance task

Initial studies, not shown, indicated that chicks could tolerate injection loads of up to 10 μg of antibodies/hemisphere without suffering overt physiological or behavioural stress. There was no effect of the antibody on the chick's behaviour during pretraining and training on the task. However, as Fig. 2 shows, chicks injected bilaterally with 1 μg or 5 μg of mAb22C11/hemisphere 30 min before training showed significant amnesia when tested 30 min later. Amnesia persisted for at least the subsequent 24 h and was not apparent if the antibody was injected just post-training or 5.5 h after training (data not shown).

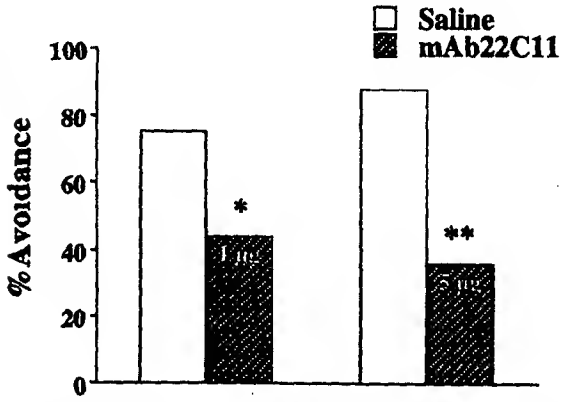

FIG. 2. Effect of mAb22C11 on retention of training. Chicks were trained and tested as described in Materials and Methods. Thirty minutes pretraining, chicks were injected bilaterally either with saline or 1 or 5 μg/hemisphere of mAb22C11, and tested for retention 30 min post-training (16–20 chicks/group). Injections of 1 and 5 μg/hemisphere of anti-βAPP 30 min pretraining resulted in amnesia when analysed by *g*-test (1 μg/hemisphere, $g = 5.175$, $P < 0.025$; 5 μg/hemisphere, $g = 9.124$, $P < 0.005$).

Effects of βAPP antisense on βAPP expression, learning and memory for the passive avoidance task

Dose-dependence

Using 16-mer end-protected phosphodiester oligodeoxynucleotide designed to correspond to the –146 and to AUG_{1786} transcription start site, we determined whether antisense-mediated downregulation of βAPP might affect memory formation. Increasing amounts of antisense oligodeoxynucleotides (AS ODNs) (0.6–1 μg/hemisphere) were injected intracerebrally at 12 h pretraining and chicks were tested at different times post-training. Controls were treated with scrambled (SC) ODNs or saline and trained and tested as the AS ODN-treated groups. As Fig. 3 shows, when compared with animals treated with scrambled ODNs, memory retention was significantly reduced when tested 24 h later in animals injected with 0.8 μg or 1 μg of antisense ODNs. The slight retention deficit resulting from injection of scrambled ODNs was not statistically significant when compared with saline-injected controls.

Time-dependence

Figure 4 shows that amnesia was produced only if AS ODNs were injected 6 or 12 h before training (group A and B) and that under such conditions amnesia was evident within 30 min post-training and lasted for at least the subsequent 24 h. In contrast, injections of AS ODNs 30 min before the training (group C) had no effect on memory consolidation and produced no amnesia.

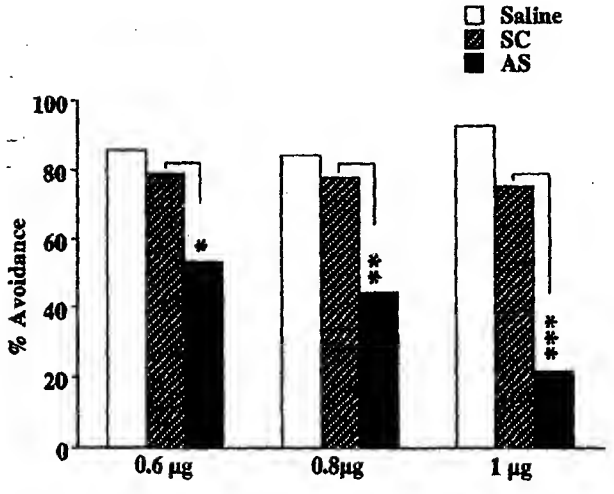

FIG. 3. Effect of increasing amounts of βAPP ODNs on memory retention. Chicks (12–16-h-old; 16–20 chicks/group) were injected intracranially with either saline or 0.6, 0.8 or 1 μg in 2 μL in each hemisphere of ODNs 11–12 h before training. Effect on memory retention was tested 30 min after training. Differences in retention scores between SC and AS ODN-treated animals were analysed using a *g*-test (0.6 μg/hemisphere, $g = 8.7562$, $P < 0.05$; 0.8 μg/hemisphere, $g = 10.307$, $P < 0.01$; 1 μg/hemisphere, $g = 17.5388$, $P < 0.001$).

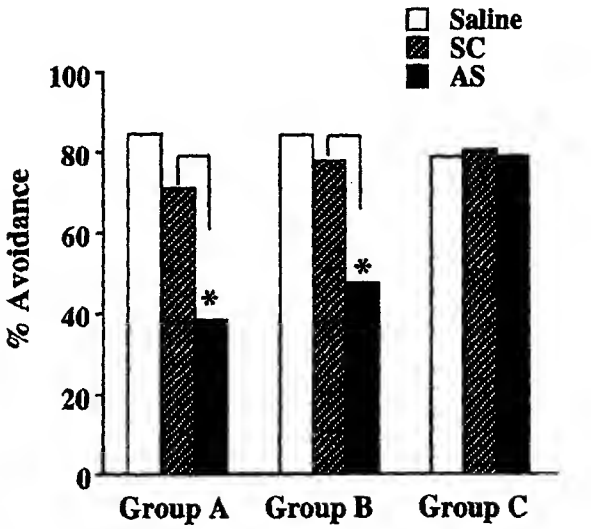

FIG. 4. Time of onset of amnesia for a passive avoidance task in chicks injected with ODNs. Chicks (12–16-h-old; 16–20 group) were injected intracranially with either saline or 0.6, 0.8 or 1 μg in 2 μL in each hemisphere of ODNs 11–12 h (group A), 6 h (group B) and 30 min before training (group C). Retention was tested 30 min and 5.5. h after training. Retention scores between groups were analysed using a *g*-test. (12 h before training, $g = 4.592$, $P < 0.05$; 6 h before training, $g = 5.020$, $P < 0.05$).

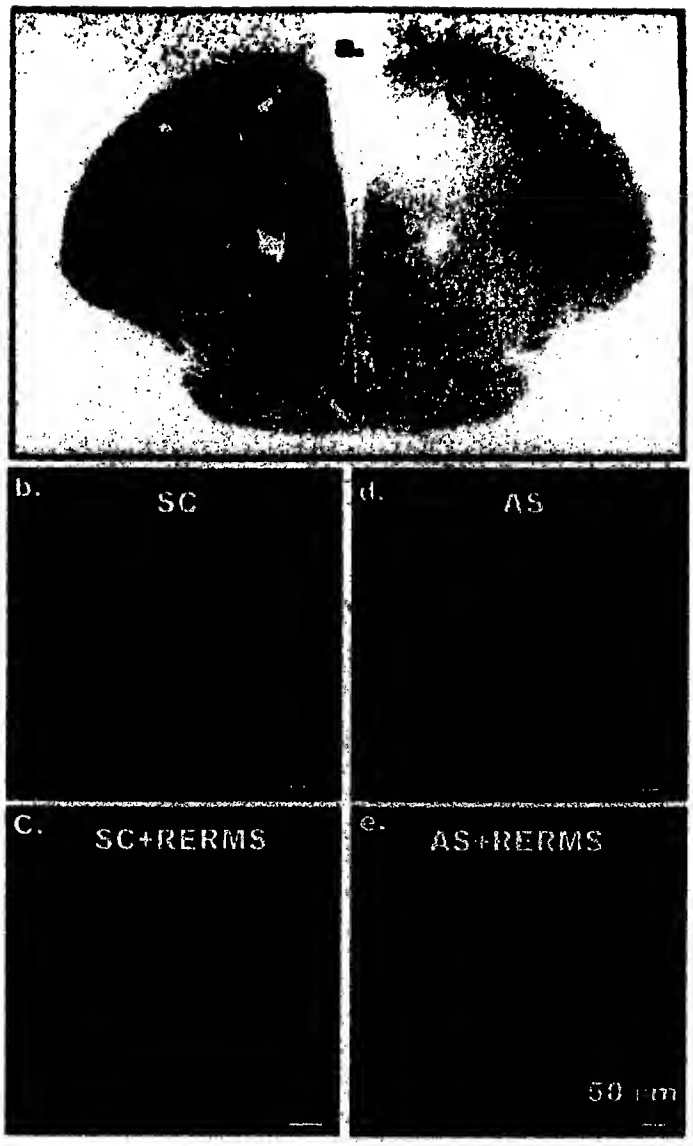

FIG. 5. Expression of βAPP in chick brain after ODNs and RERMS treatment. Chicks (12–16-h-old; 16–20/group) were injected intracranially with either 0.8 µg in 2 µL in each hemisphere of ODNs or 2 µg in 2 µL in each hemisphere of RERMS peptide. Chicks injected with saline, SC ODNs and RERMS received a second injection of saline 30 min pretraining, whilst chicks pretreated with AS ODNs were re-injected with RERMS peptide 30 min pretraining. The effect on memory retention was tested 30 min after training. Animals were decapitated immediately after testing, and *in situ* hybridization and immunocytochemistry was carried out as described in Materials and Methods. (a) Autoradiograph of *in situ* hybridization (arrow indicating injection sites); Texas red-conjugated antimouse IgG was used to detect the βAPP. (b and d) ODN treatment; (c and e) ODNs + RERMS treatment.

TABLE 1. Effects of RERMS peptide on memory retention

	Avoidance (%)					
	−2 h	−1 h	−30 min	+30 min	+1 h	+ 2 h
Saline	78	81	79	78	82	85
RERMS	75	75	76	80	78	81

There were no significant differences between RERMS and saline results ($P > 0.05$).

In order to demonstrate whether AS ODNs, injected under conditions in which they affect memory retention, suppress βAPP-mRNA and/or APP protein expression, animals were injected with 0.8 µg of βAPP antisense and their brains were analysed by *in situ* hybridization and imunocytochemistry. AS treatment, as compared to the SC controls, downregulated mRNA expression (Fig. 5a) as well as APP protein expression (Fig. 5d and e) in brain sections taken around the injection site in the IMHV.

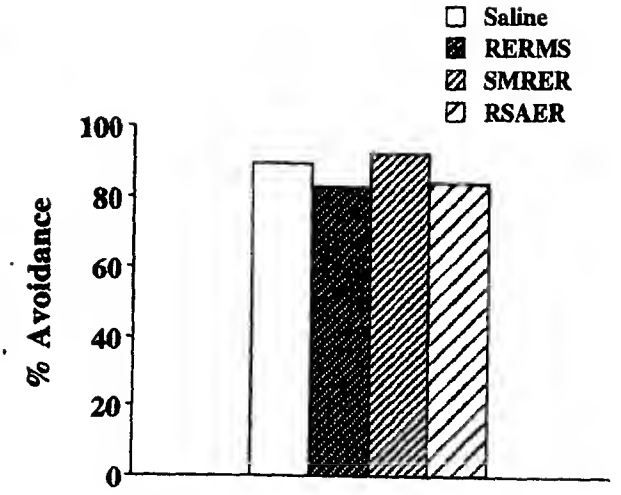

FIG. 6. Effect of APP328–332 peptides on memory. Chicks (12–16-h-old; 16–20/group) were injected intracranially with either saline or APP peptides (3 μg in 2 μL in each hemisphere) 30 min pretraining. The effect on memory retention was tested 30 min after training. Retention scores between groups were compared by *g*-test.

Effect of administration of RERMS, SMRER and RSAER peptide on learning and memory for the passive avoidance task

Initial studies indicated that chicks could tolerate intracerebral injection loads of up to 5 μg of pentapeptides without suffering overt physiological or behavioural stress(data not shown) and that RERMS peptide (2 μg peptide/hemisphere) injected at different time points before or after training had no effect on the chick's memory retention when tested 24 h later (Table 1). As RERMS seems to be a unique to APP and critical for neurite extension, cell-surface binding and inositol polyphosphate accumulation (Ninomiya *et al.*, 1993; Jin *et al*, 1994), the reverse-sequence peptide (SMRER) and the scrambled-sequence peptide with single aminoacid substitution (RSAER; Met/Ala) were used as appropriate controls. As Fig. 6 shows there was no effect of the pentapeptides (2 μg peptides/hemisphere injected 30 min before training) on the chick's memory retention when tested 30 min later.

Effect of administration of RERMS, SMRER and RSAER peptide to antisense-treated animals

We next determined whether the synthetic RERMS pentapeptide might have a potential protective effect against the memory deficit induced by downregulation of βAPP expression. Administration of 2 μg/hemisphere of RERMS prevented the memory deficit caused by βAPP antisense when injected 30 min pretraining (Fig. 7a). The reverse sequence, which had no activity in the fibroblast growth and neurite extension assay, had a substantial effect on memory rescue (Fig. 7b). By contrast, the pentapeptide in which methionine was replaced by alanine (RSAER) had no effect. Injection of peptides at different time-points prior to (1, 2 and 4 h) or after (30 min, 1, 3 and 5.5 h) training had no detectable protective effect on AS-induced amnesia (data not shown). Moreover, neither RERMS nor SMRER themselves affected retention in this task. To confirm that RERMS itself was without a memory-enhancing effect it was injected at the same dose (2 μg/hemisphere) 30 min before training the chicks on a weak version of the passive avoidance task in which agents such as

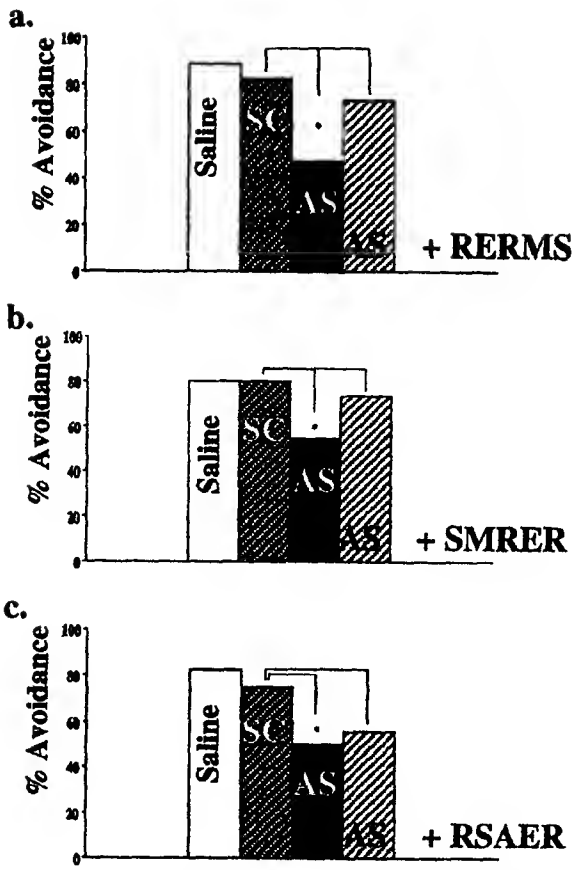

FIG. 7. Effect of APP328–332 peptides on memory rescue.Chicks (12–16-h-old; 16–20/group) were injected intracranially with either saline, APP peptides (2 μg in 2 μL in each hemisphere) or 0.8 μg in 2 μL in each hemisphere of ODNs. Chicks injected with saline, SC ODNs and RERMS received a second injection of saline 30 min pretraining, whilst chicks pretreated with AS ODNs were re-injected with APP peptide 30 min pretraining (16–20 chicks/group). The effect on memory retention was tested 30 min after training. Retention scores between groups were compared by *g*-test. (a) AS + RERMS treated animals, $g = 8.756$, $P < 0.005$); (b) AS + SMRER treated animals, $g = 10.18$, $P < 0.005$; (c) AS + RSAER treated animals, $g = 2.854$, $P < 0.1$).

corticosterone (Sandi & Rose, 1994) or BDNF (Johnston *et al.*, 1999) will enhance 24 h retention. RERMS was without memory-enhancing effect (data not shown).

To determine whether APP protein expression in ODNs treated animals was affected by APP328–332 treatment, brains were analysed by immunocytochemistry as shown in Fig. 5c. RERMS treatment had no visible effect on APP distribution as compared to AS treatment (Fig. 5e).

Discussion

Over the past 10 years there has been a remarkable convergence of evidence pointing to a key role for cell adhesion molecules in the sequence of restructuring of synaptic connectivity (for review see Rose, 2000). The argument that the amyloid precursor protein might also be involved in this process is strengthened by evidence that APP modulates a variety of responses in neurons, including stimulation of

neurite outgrowth (Mattson, 1994; Storey *et al.*, 1996), promotion of synaptogenesis (Mucke *et al.*, 1994; Roch *et al.*, 1994), modulation of synaptic plasticity (Doyle *et al.*, 1990; Huber *et al.*, 1993, 1997; Roch *et al.*, 1994; Ishida *et al.*, 1997), suppression f the Ca^{2+} response to glutamate and protection of neurons against excitotoxic damage (Mattson *et al.*, 1993a, b; Goodman & Matson, 1994; Goodman & Mattson, 1996). Therefore, it has become important to assess the putative role of βAPP in memory formation itself.

The role of APP in memory formation has been attributed to its involvement in cell-to-substrate adhesion processes. The data reported in this paper suggests that the APP involvement in memory formation most probably involves change in signal transduction events. The post-training time within which the antibody and antisense-induced amnesia, and within which RERMS and SMRER prevents amnesia, corresponds to that during which memory formation is vulnerable to disruption of the putative signal-transduction functions of APP.

The chick system is a good one for exploring these issues, because the learning task is precise and sharply timed, and permits one also to be sure that any observed effect of an injected substance is specific to ntion and not either to acquisition or to concomitant processes such as visual acuity, arousal or motor activity (Rose, 2000). Further, the role of other cell adhesion molecules in the cascade leading to synaptic modulation has been well mapped, so that the effects of either blocking or attempting to rescue functional APP activity can be set into an established context (For review see Rose, 2000).

It was, however, first necessary to demonstrate that the antibody to mouse N-terminal domain of APP did recognize an epitope in the chick. As Fig. 1a demonstrates, chick SPM contains a protein with a similar molecular weight to that of mammalian APP that cross-reacts with the mouse antibody. The localization of the protein recognized by the anti-APP in chick IMHV is revealed by the immunocytochemical labelling shown in Fig. 1b–d. When injected into the IMHV, mAb22C11 showed strong immunofluorescence with extensive binding to neuropil and around the ventricle in the minutes after injection (Fig. 1h and i). However, within 60 min postinjection most fluorescence was concentrated around the ventricles, and by 6 h had almost entirely disappeared. This is comparable with the residence times found, for instance, for antibodies to NCAM (Scholey *et al.*, 1995), and contrasts with the much briefer time we have found for ntibodies to ApoE (Lancashire *et al.*, 1998). The residence time for anti-APP coincides with the relatively rapid turnover time for membrane-bound APP (20–60 min; Storey *et al.*, 1996).

Training day-old chicks on a one-trial passive avoidance task results in a time-dependent cellular and molecular cascade, which involves two distinct waves of protein synthesis, as evidenced by the effects of anicomycin, 2-deoxygalactose, anti-L1 and anti-NCAM on memory formation (Rose, 1995, 1996). Therefore, in this study we focused on the two time points relative to training that were extensively investigated in our previous studies: 30 min pretraining and 5.5 hr post-training. mAb22C11 pretraining did not affect behaviour during pretraining, or interfere with the chicks' pecking at the bitter bead and learning the avoidance task; however, it did result in amnesia in birds tested 30 min later (Fig. 2). The fact that pretraining administration of antibody resulted in amnesia apparent within 30 min suggests that the N-terminal domain of APP is required at an early phase of memory consolidation, rather than in the transition to long-term memory which we have postulated for NCAM and L1 (Rose, 1995; Scholey *et al.*, 1995). The results described above suggest that the amnesia caused by mAb22C11 antibody administration occurs either an early phase of synaptic remodelling requiring cell–matrix interaction or a putative signal transduction function of APP, which interferes with the cascade of synaptic transients required to initiate memory formation. However, it remains the case that receptor-mediated APP-induced signal transduction has not been fully established, despite the demonstration f specific binding sites in neural cells (Jin *et al.*, 1994) and vidence that sAPP affects neuronal cells in picomolar concentrations (Mattson *et al.*, 1993b).

A more direct approach to interfering with the functioning of the protein during the processes of training and recall is provided by the use of antisense (Mileusnic *et al.*, 1996; Mileusnic *et al.*, 1999; Mileusnic, 1999). Due to the apparent toxicity of nuclease-resistant phosphorothioate and methylphosphonate found in preliminary experiments, as well as because of the phosphorothioate interaction with basic proteins, end-protected ODNs were used. There was no effect of administering these ODNs on chick behaviour during pretraining or training on the task. As shown in Figs 3 and 4, injecting antisense ODNs had marked effects on memory formation. *In situ* hybridization and immunocytochemistry (Fig. 5) showed that the antisense did indeed decrease APP gene expression.

βAPP is a multifunctional protein involved in neuronal growth, survival and differentiation. The distinct differences in the concentration dependence for these three different activities suggest that different molecular mechanisms may be involved in the three APP functions (Yamamoto *et al.*, 1994). Saitoh and collaborators mapped the growth-promoting activity of sAPP to a domain of the five amino acid sequence RERMS (APP328–332) and showed that peptides containing the RERMS sequence bind to the specific membrane sites on the cell surface (Jin *et al.*, 1994). In the present study, we asked whether this sequence of five amino acids was also active in rescuing the memory lost following antisense treatment. Indeed, pretraining injection of 2 μg/hemisphere of the APP328–332 (RERMS) protected the memory against antisense-induced loss (Fig. 6). Furthermore, the experiments to test the sequence specificity of the 5-mer pepide have shown that the reverse-sequence peptide (SMRER), which had no activity in fibroblast growth assay but showed a substantial effect on supporting survival of the primary neuronal culture (Yamamoto *et al.*, 1994), was also active in rescuing antisense-induced memory loss. Several lines of evidence suggested that reduction of APP gene expression results in reduction in cell proliferation, autocrine regulation of cell growth and cell adhesion (Saitoh *et al.*, 1989; Schubert *et al.*, 1989; Ueda *et al.*, 1989) as well as marked induction of neurite extension (LeBlanc *et al.*, 1992; Schubert & Behl, 1993). One possible mechanism of APP328–332 action might therefore be its effect on intracellular signal transduction through interaction with cell-surface receptors. A scrambled peptide sequence with single amino acid substitution in the APP328–332 peptide (RSAER) was sufficient to abolish the effect on antisense-induced amnesia. This finding argues for the mechanism of APP328–332 effect in abolishing memory disruption caused by βAPP antisense treatment by some process other than cell adhesion. The mechanisms by which RERMS and SMRER peptides abolish the antisense-induced amnesia remain to be elucidated.

Results reported in this paper indicate that APP has multiple and important functions and plays an essential part in the biochemical cascade leading to memory consolidation. The timecourse of onset of amnesia that occurs as a result of blocking the N-terminal external domain of APP might suggest that an impaired adhesive function of APP could lead to the impaired transmisional configuration of the synapses and to disruption of the processing of its external domains in the synaptic cleft, thus contributing to the observed memory dysfunction. However, the observation that APP328–332 reversed the effect of APP-antisense-induced amnesia supports the notion that

APP is one of the substrate adhesion molecules (SAMs) that exerts its effects on intracellular signal transduction involved in the cascade of synaptic transients required to initiate memory formation.

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Abbreviations

Aβ, β-amyloid; APP, amyloid precursor protein; AS ODNs, antisense oligodeoxynucleotides; AS, antisense; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IMHV, intermediate medial hyperstriatum ventrale; MeA, methylanthranilate; NeuN, neuron-specific nuclear protein; RERMS, NH_2-Arg-Glu-Arg-Met-Ser-COOH; sAPP, secreted form of APP; SC, scrambled; SPM, synaptic plasma membrane.

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